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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>A61K 39/012</b>	A1	(11) International Publication Number: <b>WO 96/40234</b> (43) International Publication Date: 19 December 1996 (19.12.96)
(21) International Application Number: <b>PCT/IB95/00446</b>		(81) Designated States: CA, FI, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date: 7 June 1995 (07.06.95)		
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(54) Title: IN OVO VACCINATION AGAINST COCCIDIOSIS

(57) Abstract

The invention relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo* an effective immunizing dose of live *Eimeria* sporozoites or merozoites, or a mixture thereof. In a preferred embodiment, the domesticated bird that is vaccinated is a chicken or turkey.

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IN OVO VACCINATION AGAINST COCCIDIOSISBackground of the Invention

The present invention relates to a method of vaccinating domesticated birds against coccidiosis. In particular, the invention relates to the *in ovo* administration of live *Eimeria* spp sporozoites or merozoites, or mixtures thereof, into the developing 10 eggs of domesticated birds in order to immunize the hatched chicks against coccidiosis.

Coccidiosis is an enteric disease of domesticated birds caused by infection with intracellular protozoan parasites of the genus *Eimeria*. Coccidiosis is the most 15 economically devastating parasitic disease of domesticated birds. It is estimated that anticoccidial medications and losses due to coccidiosis cost the poultry industry hundreds of millions of dollars every year.

Various attempts to vaccinate domesticated birds against coccidiosis have been reported since the early 1950's. Current vaccination methods include administering live *Eimeria* oocysts to birds through feed or water. These methods, 20 however, are inconvenient and inefficient because not all birds get the intended oocyst dose and many are either unprotected by the vaccine or receive a pathogenic infection.

In J. M. Sharma and B. R. Burmester, *Avian Dis.* 26: 134-149, 1981, the authors reported that chickens vaccinated *in ovo* with herpesvirus of turkey 25 developed immunity against subsequent challenge with Marek's disease virus. In European patent publication no. 291173, an immunization process is referred to wherein a nonreplicating immunogen is administered *in ovo*. The immunogens specifically referred to in the European patent are a genetically engineered *Eimeria* antigen and an *Eimeria* oocyst extract. The European patent specifically excludes 30 live parasite stages such as those used in the vaccination method claimed herein.

The present vaccination method involves *in ovo* administration of live *Eimeria* sporozoites or merozoites, or a mixture thereof, into the developing eggs of domesticated birds. The available literature suggests that such a vaccination method would be ineffective *in ovo* and should be applied post-hatch. In T.K. Jeffers and 35 G.E. Wagenbach, *J. Parasit.* 56(4): 656-662, 1970, the authors reported that *in ovo* injection of *E. tenella* sporozoites on day 10 of incubation provided no significant immunological protection against subsequent challenge with *E. tenella* oocysts. In

fact, they reported that chicks that received no treatment had a greater survival rate against subsequent challenge with *E. tenella* oocysts than chicks that had been treated *in ovo* with sporozoites. In K.L. Watkins et al., Proc. VI th. International Coccidiosis Conf., Abstract E1-2, Ontario, Canada, 1993, the authors described *in ovo* inoculation with live *E. maxima* sporocysts and sporulated oocysts and concluded that their study provided no evidence that *in ovo* exposure protects against subsequent coccidial challenge with *E. maxima* oocysts 10 days post-hatch. Watkins et al. further concluded that significant immunological protection is provided if inoculation is done soon after hatch rather than *in ovo*. Contrary to this teaching, 10 the *in ovo* vaccination method of the present invention provides unexpected immunity that protects the hatched birds against subsequent coccidial challenge.

#### Summary of the Invention

The present invention, also referred to herein as the "present vaccination method", relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo*, during the final quarter of incubation, an effective immunizing dose of live *Eimeria* sporozoites or merozoites, or a mixture thereof.

The term "domesticated bird(s)", as used herein, unless otherwise indicated, includes chickens, turkeys, ducks, game birds (including, but not limited to, quail, 20 pheasants, and geese) and ratites (including, but not limited to, ostrich).

The term "*in ovo*", as used herein, unless otherwise indicated, means into a domesticated bird egg containing a live, developing embryo.

The term "administering *in ovo*" or "*in ovo* administration", as used herein, unless otherwise indicated, means administering the vaccine described herein to a 25 domesticated bird egg containing a live, developing embryo by any means of penetrating the shell of the egg and introducing the vaccine. Such means of administration include, but are not limited to, injection of the vaccine.

The term "final quarter of incubation", as used herein, unless otherwise indicated, means the final quarter of incubation of a developing egg of a 30 domesticated bird.

The term "*Eimeria*", as used herein, unless otherwise indicated, means one or more species of the genus *Eimeria* that infect domesticated birds. Such *Eimeria* species include those that are found in chicken, including *E. tenella*, *E. acervulina*,

*E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*, and also those that are found in turkeys, including *E. meleagrinitis*, *E. adenooides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*, and also *Eimeria* species that infect other domesticated birds as defined above. The term "*Eimeria*" also includes

5 all strains of the foregoing species of *Eimeria*, including, but not limited to, precocious strains, and attenuated strains, which includes strains that have been irradiated, or otherwise treated, so that they fail to complete development. The term *Eimeria* also includes any newly-discovered strains or species of *Eimeria* that infect domesticated birds as defined above.

10 The terms "sporozoites", "sporocysts", "oocysts", and "merozoites", as used herein, unless otherwise indicated, mean live *Eimeria* sporozoites, sporocysts, oocysts, and merozoites.

15 The term "effective immunizing dose", as used herein, unless otherwise indicated, means a number of sporozoites or merozoites, or, when mixed, a number of sporozoites and merozoites, sufficient to provide immunological protection in the hatched birds that is greater than the inherent immunity of non-immunized birds. As used herein, the terms "immunize" and "vaccinate" are synonymous and are used interchangeably.

20 A preferred dose to be administered in accord with the method of the invention comprises 10 to  $10^6$  sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10 to  $10^6$ .

25 A more preferred dose comprises  $10^3$  to  $10^6$  sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from  $10^3$  to  $10^6$ .

Another preferred dose comprises  $10^2$  to  $10^5$  sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from  $10^2$  to  $10^5$ .

30 A preferred domesticated bird to be vaccinated in accord with the method of the invention is a chicken.

A preferred dose to be administered *in ovo* to chicken eggs comprises sporozoites or merozoites, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*. The dose preferably includes from 10

to  $10^6$  sporozoites or merozoites, or a mixture thereof, for each species that is included in the dose.

Another preferred domesticated bird to be vaccinated in accord with the method of the invention is a turkey.

5 A preferred dose to be administered *in ovo* to turkey eggs comprises sporozoites or merozoites, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. meleagrinitis*, *E. adenoeides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*. The dose preferably includes from 10 to  $10^6$  sporozoites or merozoites, or a mixture thereof, 10 for each species that is included in the dose.

Other preferred domesticated birds to be vaccinated in accord with the method of the invention are game birds, ducks and ratites.

15 The method of the invention further comprises, in combination with the present vaccination method, administering *in ovo* an immune stimulant at any time during incubation.

A preferred method of administering the immune stimulant is simultaneously with the *in ovo* administration of a dose of sporozoites or merozoites, or mixture of said sporozoites and merozoites, during the final quarter of incubation.

20 Detailed Description of the Invention

The present vaccination method involves the *in ovo* administration, during the final quarter of incubation, of live *Eimeria* sporozoites or merozoites, or a mixture thereof, into domesticated birds' eggs. In the case of chickens, *in ovo* administration is preferably done on days 15-20 of incubation, and most preferably on day 18 of 25 incubation. In the case of turkeys, *in ovo* administration is preferably done on days 21-26 of incubation.

The present vaccination method can be performed using any suitable *in ovo* administration method. Preferably, the present vaccine is administered via injection. According to one method of injection, a hole is made in the egg shell at the large end 30 of the egg using an 18 gauge needle to expose the egg's air cell. A 1.0-1.5 inch 22 gauge needle attached to a syringe of appropriate size (1-3 ml) can be inserted through the hole and through the membrane of the air cell. An appropriate number of sporozoites or merozoites, or, when mixed, an appropriate number of sporozoites

and merozoites, are suspended in a suitable liquid carrier, for instance 10-500 $\mu$ l of phosphate-buffered saline, and then injected into the egg. The appropriate volume will depend on the size of the egg being treated, with ostrich eggs obviously being capable of taking more volume than chicken eggs. The site of injection can be within

5 any region of the egg or embryo. Preferably, injection is done axially through the center of the large end of the egg into the amnion.

Alternatively, an automated egg injection system can be used in the present vaccination method. Such systems are described in U.S. Patent nos. 4,681,063, 4,040,388, 4,469,047, and 4,593,646, which are herein incorporated by reference.

10 Other appropriate methods of injection are known to those skilled in the art.

Oocysts can be prepared by any of several methods known to those skilled in the art. Such methods include those described in J.F. Ryley et al., *Parasitology* 73:311-326, 1976 and P.L. Long et al., *Folia Veterinaria Latina* VI#3, 201-217, 1976, which are herein incorporated by reference. According to one method,

15 commercial broiler chickens, approximately 2 weeks old, are infected with the *Eimeria* species of interest by oral gavage of an appropriate dose of sporulated oocysts. For example, a typical dose used for *E. tenella* is 200,000 sporulated oocysts/bird. Well known procedures for collection and purification of oocysts from infected birds are then followed. For most species of *Eimeria*, feces are collected from infected birds

20 5-7 days post-infection, blended and filtered to remove debris, then centrifuged at a speed sufficient to pellet the remaining fecal material. For *E. tenella*, a similar procedure is used except that cecal cores are taken at 6 days post-infection. The pellet is resuspended in a saturated salt solution, in which the oocysts float and most of the contaminating debris can be removed by centrifugation. The oocyst suspension is then diluted to lower the salt concentration. The oocysts are washed

25 repeatedly to remove the salt and resuspended in potassium dichromate solution (2.5% w/v). The oocyst suspension is incubated at 29°C with shaking (e.g., 140 rpm) for approximately 72 hours to induce sporulation of the oocysts. Alternatively, the oocysts can be treated with sodium hypochlorite and then sporulated. The

30 number of sporulated oocysts/ml is determined by direct count using a hemocytometer, and the culture is stored, preferably under refrigeration until needed.

To prepare sporocysts, the potassium dichromate is removed from the oocyst suspension described above by repeated washing of the oocysts, which involves collection of oocysts by centrifugation and resuspending in deionized or distilled water. When the dichromate has been removed as judged by the lack of yellowish-

5 orange coloration, the oocyst suspension is mixed with an equal volume of sodium hypochlorite (bleach) and incubated at room temperature for 15 minutes. The bleach is then removed by repeated washings, and the oocysts are resuspended in physiological saline or deionized water. Oocysts can be broken to release sporocysts using a variety of known techniques. For example, oocysts can be broken to release

10 sporocysts by mixing the oocysts with glass beads of 1-4 mm diameter and shaking by hand, vortex mixer, or shaking incubator, or using a hand-held homogenizer. Unbroken oocysts and oocysts walls can be separated from the released sporocysts by differential centrifugation in 50% Percoll™ (sold by Pharmacia Biotech) or 1 M sucrose as described in Dulski et al., Avian Diseases, 32: 235-239, 1988.

15 To prepare sporozoites or a sporozoite-rich preparation to be used in accord with the present invention sporozoites are excysted from the sporocyst preparation described above. In one procedure, sporocysts prepared as described above are pelleted by centrifugation, resuspended in excystation buffer (0.5% taurodeoxycholic acid and 0.25% trypsin in phosphate buffered saline, pH 8.0) and incubated with

20 shaking for one hour at 41 °C. A sample of the resulting suspension is counted to determine the number of sporozoites, the sporozoites are washed once to remove the excystation buffer, and resuspended in phosphate-buffered saline at the desired concentration for *in ovo* injection. This preparation contains sporozoites, sporocysts and oocysts, and, without further purification, can be used in accord with the

25 present vaccination method. Purified sporozoites, removed from sporocysts and oocysts, can be prepared by DE-52 anion exchange chromatography as described in D.M. Schmatz et al., J. Protozool. 31: 181-183, 1984, which is herein incorporated by reference. The dose of sporozoites to be used in the present vaccination method will vary according to the type of domesticated bird being vaccinated and the species

30 of *Eimeria* being used in the vaccine. In general, the dose can range from 10 to  $10^6$  sporozoites per egg. Preferably, the dose ranges from 10 to  $10^5$  sporozoites per egg, and, more preferably, the dose ranges from  $10^2$  to  $10^5$  sporozoites per egg.

Merozoites can be prepared by various methods known to those skilled in the art. In one method, sporozoites are infected into primary chick kidney cells (PCK) that are grown in culture as cell aggregates, using a modification of the method described in D. J. Doran, J. Parasit. 57: 891-900, 1971, which is herein incorporated by reference. PCK cells are grown at 40°C in 3% CO<sub>2</sub> in modified RK2 medium - DMEM/F12 with L-glutamine and 15 mM HEPES, supplemented with fetal bovine serum, penicillin-streptomycin, 15 mM sodium bicarbonate, 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid and 0.01 µM hydrocortisone HCL as described in S.D. Chung et al., J. Cell Biol. 95: 5 118-126, 1982. PCK cells are prepared from kidneys of 2 to 3 week old chicks by mincing the kidney, and then treating the tissue at 37°C with several changes of 0.2 mg/ml collagenase (sold by Worthington Biochemical Corp., Freehold, NJ) in phosphate-buffered saline solution. The cellular aggregates in the supernatant are washed, resuspended in modified RK2 medium containing 5% fetal bovine serum and 10 15 used to seed tissue cultures flasks at a density of 10<sup>6</sup> aggregates per cm<sup>2</sup>. The PCK cells are incubated for 18 hours at 40°C in 3% CO<sub>2</sub> and then infected with 4 x 10<sup>6</sup> sporozoites/cm<sup>2</sup>. Infected cultures are grown in modified RK2 medium containing 2% fetal bovine serum. After 24 hours of incubation, to allow invasion, uninvaded sporozoites are removed by agitating the flask and removing the culture medium. 20 The cell layer is washed once with modified RK2 medium containing 2% fetal bovine serum and the culture medium is again discarded. Fresh RK2 medium is added, and the cultures are incubated for another 48 to 54 hours until merozoites are released into the culture medium.

Purification of the merozoites to remove host cell debris can be done by 25 various methods known to those skilled in the art. In accord with one method, as described in J.A. Olson, Antimicrob. Agents Chemother. 34: 1435-1439, 1990, the culture medium containing the released merozoites is collected and spun at 450 X g for 10 minutes to concentrate the merozoites. The pellet containing the merozoites and the host cell debris is suspended in 0.1 M NaCl-0.05 M KCl-20% 30 bovine serum albumin and applied to a DE-52 anion exchange column equilibrated in 75 mM Tris-40 mM NaH<sub>2</sub>PO<sub>4</sub>-86 mM NaCl-100 mM glucose at pH 8.2. Merozoites flow through the column. Merozoites collected from the column can be tested for purity by electron microscopy as described in A. Kilejian, J. Biol. Chem. 249: 4650-

4655, 1974. In general, the merozoite dose can range from 10 to  $10^6$  merozoites per egg. Preferably, the dose ranges from 10 to  $10^5$  merozoites per egg, and, more preferably, the dose ranges from  $10^2$  to  $10^5$  merozoites per egg. When sporozoites and merozoites are mixed, in general, the dose comprising the total number of 5 merozoites and sporozoites can range from 10 to  $10^6$  per egg. Preferably, the dose ranges from 10 to  $10^5$  merozoites and sporozoites per egg, and, more preferably, the dose ranges from  $10^2$  to  $10^5$  merozoites and sporozoites per egg.

The sporozoites or merozoites, or mixture thereof, can be injected *in ovo* in any physiologically suitable medium. Preferably, they are suspended in 10 physiologically balanced saline such as phosphate-buffered saline. The selected medium can optionally include one or more suspending agents including physiologically suitable gels, gelatins, hydrosols, cellulose, or polysaccharide gums.

15 Preferably, in the present vaccination method, sporozoites or merozoites, or a mixture thereof, of two or more *Eimeria* species are injected *in ovo* at the same time. In accord with the present vaccination method, sporozoites or merozoites, or a mixture thereof, of all identified species of *Eimeria* that infect a specific domesticated bird, such as chicken, can be injected *in ovo* at the same time, or in series, at appropriate doses to provide immunological protection against all species.

Immune stimulants can be used in conjunction with the present vaccination 20 method. Suitable immune stimulants include, but are not limited to, cytokines, growth factors, chemokines, supernatants from cell cultures of lymphocytes, monocytes, or cells from lymphoid organs, cell preparations or cell extracts (e.g. *Staphylococcus aureus* or lipopolysaccharide preparations), mitogens, or adjuvants including low molecular weight pharmaceuticals. An immune stimulant can be 25 administered *in ovo* at any time during incubation. Preferably, an immune stimulant is administered *in ovo* in the medium containing the dose of *Eimeria* sporozoites or merozoites, or mixture thereof.

The efficacy of the present invention in vaccinating against coccidiosis is 30 illustrated in the following examples. Each dose was injected *in ovo* in physiologically-acceptable saline as described above. The effectiveness of a particular preparation was determined by monitoring its effect on hatch rate and hatch weight of the chicks, and, following challenge infection, oocyst production, weight gain, and pathogenicity (lesion score). Lesion scores were assigned

-9-

according to the protocol described in J.K. Johnson and W.M. Reid, *Exp. Parasitol.* 28: 30-36, 1970, according to which a value of 0 represents no disease and a value of 4 represents maximum pathology.

5

EXAMPLE 1

Chicken eggs were injected on day 18 of incubation with a preparation containing  $10^5$  *E. tenella* sporozoites per egg. The preparation was not purified to remove sporocysts and oocysts. Each dose also contained approximately  $10^4$  *E. tenella* sporocysts and approximately  $10^4$  *E. tenella* oocysts. As a control, eggs were 10 injected only with phosphate-buffered saline solution. In the sporozoite-treated population of birds the mean oocyst shed at 7 days post-hatch was  $1.1 \times 10^6$  oocysts/bird. Non-immunized birds and the sporozoite-treated birds were challenged with various doses of sporulated oocysts of *E. tenella*, administered by oral gavage, on days 7, 14 or 21 post-hatch. The data appear in Table 1.

15

-10-

## Immunized Versus Non-Immunized Birds: Responses To Different Challenge Doses At Different Times Post-Hatch

TABLE 1

Group <sup>1</sup>	Lesion score on sixth day after challenge		Weight gain (grams) per bird over six day period after challenge
	Challenge dose (sporulated oocysts per bird)	Non-immunized	
Challenge on day 7 post-hatch	0	0	188
	$2.5 \times 10^3$	3.0	0.3*
	$5 \times 10^3$	3.1	1.2*
	$1 \times 10^4$	3.7	1.4*
	0	0	0
	$2.5 \times 10^3$	2.4	0.4*
	$5 \times 10^3$	2.8	1.1*
	$1 \times 10^4$	3.3	1.6*
	0	0.1	0
	$2.5 \times 10^3$	2.3	0.7*
	$5 \times 10^3$	2.1	0.8*
	$1 \times 10^4$	2.8	1.4*
Challenge on day 14 post-hatch	0	0	167
	$2.5 \times 10^3$	3.0	0.3*
	$5 \times 10^3$	3.1	1.2*
	$1 \times 10^4$	3.7	1.4*
	0	0	153
	$2.5 \times 10^3$	2.4	0.4*
	$5 \times 10^3$	2.8	1.1*
	$1 \times 10^4$	3.3	1.6*
	0	0.1	0
	$2.5 \times 10^3$	2.3	0.7*
	$5 \times 10^3$	2.1	0.8*
	$1 \times 10^4$	2.8	1.4*
Challenge on day 21 post-hatch	0	0	167
	$2.5 \times 10^3$	3.0	0.3*
	$5 \times 10^3$	3.1	1.2*
	$1 \times 10^4$	3.7	1.4*
	0	0	153
	$2.5 \times 10^3$	2.4	0.4*
	$5 \times 10^3$	2.8	1.1*
	$1 \times 10^4$	3.3	1.6*
	0	0.1	0
	$2.5 \times 10^3$	2.3	0.7*
	$5 \times 10^3$	2.1	0.8*
	$1 \times 10^4$	2.8	1.4*

1 Each group was subjected to a single challenge of sporulated oocysts on days 7, 14, or 21 post-hatch. For example, birds challenged on day 21 post-hatch were not challenged on days 7 or 14 post-hatch.

Significantly different from non-immunized birds ( $p < 0.05$ , ANOVA).

The data in Table 1 clearly show that the immunized birds were less susceptible to infection than their non-immunized hatch mates as indicated by the reduced lesion scores and improved weight gains in the immunized birds. The data 5 also demonstrate that the method of the invention imparts immunity to the chicks at a relatively early age (within seven days post-hatch). Furthermore, the data show that the immunity continues as the chicks grow and develop. Imparting immunity to chicks at an early age provides a significant advantage in the broiler chicken industry because broilers routinely reach market by 6 weeks of age.

10

EXAMPLE 2

Chicken eggs were injected on day 18 of incubation with saline (control) or preparations containing different doses of sporozoites of *E. tenella*, as indicated in Table 2 which provides the pre-challenge results. The sporozoite preparation used 15 for each dose contained 62% sporozoites, 9% sporocysts, and 29% oocysts. Each dose containing  $10^5$  sporozoites included a total of  $1.6 \times 10^5$  parasite stages (sporozoites, sporocysts, and oocysts).

20

TABLE 2

Sporozoites injected per egg on day 18 of incubation	Hatch rate (%)	Hatch weight (grams)
0	94	48.2
0	94	48.0
$10^3$	100	49.4
$10^4$	97	47.0
$10^5$	94	49.1

25

The data in Table 2 show that chicks hatched from eggs injected with live sporozoites were substantially identical to their non-immunized hatch mates in terms of hatch rate and hatch weight. The chicks were then challenged on day 14 post-

-12-

hatch with  $1.25 \times 10^4$  sporulated oocysts of *E. tenella* per bird, administered by oral gavage. The post-challenge results are provided in Table 3.

**Response To Challenge Infection Of Non-Immunized Birds Versus Birds Treated  
*In Ovo* With Different Doses Of Sporozoites**

**TABLE 3**

	Sporozoites injected per egg on day 18 of incubation	Weight Gain per bird over six day period after challenge	Lesion Score on day 6 after challenge	Oocysts shed per bird (x 10 <sup>6</sup> ) on day 6 after challenge
5	0	278	3.2	12.3
10	0 (Unchallenged control)	321	0	0.003*
15	10 <sup>3</sup>	289	2.6*	11.2
	10 <sup>4</sup>	291	2.7	12.2
	10 <sup>5</sup>	304*	1.4*	1.4*

20     Significantly different from non-immunized group (received saline) that was subjected to challenge (p<0.05, ANOVA)

25     The data in Table 3 show that for every parameter (weight gain, lesion score, oocyst shed) chicks hatched from eggs injected with different doses of the sporozoite preparation showed evidence of immunity. In comparison to the control birds that were treated only with saline and were subjected to challenge infection, the birds immunized *in ovo* with the sporozoite preparation showed greater weight gain and reduced lesion scores. In addition, the birds immunized *in ovo* with the sporozoite preparation passed fewer oocysts than the control birds after challenge infection, indicating that the infection was less severe in the immunized birds.

We claim:

1. A method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo*, during the final quarter of incubation, an effective immunizing dose of live *Eimeria* sporozoites or merozoites, or a mixture thereof.
- 5 2. The method of claim 1 wherein the dose comprises 10 to 10<sup>6</sup> sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10 to 10<sup>6</sup>.
- 10 3. The method of claim 1 wherein the dose comprises 10<sup>3</sup> to 10<sup>6</sup> sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10<sup>3</sup> to 10<sup>6</sup>.
4. The method of claim 1 wherein the dose comprises 10<sup>2</sup> to 10<sup>5</sup> sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10<sup>2</sup> to 10<sup>5</sup>.
- 15 5. The method of claim 2 wherein the domesticated bird is a chicken.
6. The method of claim 5 wherein the dose comprises sporozoites or merozoites, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*.
- 20 7. The method of claim 6 wherein the dose is administered by *in ovo* injection.
8. The method of claim 2 and further comprising administering *in ovo* an immune stimulant at any time during incubation.
- 25 9. The method of claim 8 wherein the immune stimulant is administered *in ovo* simultaneously with the dose of sporozoites or merozoites, or mixture of said sporozoites and merozoites.
10. The method of claim 2 wherein the dose comprises merozoites.
11. The method of claim 2 wherein the dose comprises sporozoites.
12. The method of claim 11 wherein the sporozoites have been purified to remove sporocysts and oocysts.
- 30 13. The method of claim 2 wherein the domesticated bird is a turkey.
14. The method of claim 13 wherein the dose comprises sporozoites or merozoites, or a mixture thereof, of two or more species of *Eimeria* selected from the

-15-

group consisting of *E. meleagrinitis*, *E. adenoeides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*.

15. The method of claim 14 wherein the dose is administered by *in ovo* injection.

5 16. The method of claim 2 wherein the domesticated bird is a game bird, duck or ratite.

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB 95/00446A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K39/012

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 291 173 (EMBREX INC.) 17 November 1988 cited in the application see page 2 - page 3 ---	1-16
A	THE JOURNAL OF PARASITOLOGY, vol. 56, no. 4, 1970 pages 656-662, T.K.JEFFERS ET AL. 'Embryonic Response to Eimeria Tenella Infection' cited in the application see abstract ---	1-16 -/-

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30 November 1995	12.01.96

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## INTERNATIONAL SEARCH REPORT

International Application No

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## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LES COLLOQUES DE L'INRA, vol. 49, 1989 pages 655-660, T.L.FREDERICKSEN ET AL. 'In Ovo administration of a potent recombinant coccidial antigen vaccine in poultry' see abstract ---	1-16
A	EP,A,0 650 733 (AMERICAN CYANAMID COMPANY) 3 May 1995 see page 2 - page 7 ---	1-16
A	WO,A,94 16725 (MERCK & CO, INC.) 4 August 1994 see page 1 - page 2 -----	1-16

## INTERNATIONAL SEARCH REPORT

## Information on patent family members

International Application No

PCT/IB 95/00446

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-291173	17-11-88	CA-A-	1326818	08-02-94
		JP-A-	1025731	27-01-89
		ZA-A-	8802708	17-10-88
EP-A-650733	03-05-95	AU-B-	7763294	18-05-95
		CA-A-	2134835	04-05-95
		JP-A-	7206705	08-08-95
WO-A-9416725	04-08-94	AU-B-	5989894	15-08-94

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